MAJOR ARTICLE

# Genetic Diversity of *Plasmodium falciparum* Histidine-Rich Protein 2 (PfHRP2) and Its Effect on the Performance of PfHRP2-Based Rapid Diagnostic Tests

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**Rising costs of antimalarial agents are increasing the demand for accurate diagnosis of malaria. Rapid diagnostic tests (RDTs) offer great potential to improve the diagnosis of malaria, particularly in remote areas. Many RDTs are based on the detection of *Plasmodium falciparum* histidine-rich protein (PfHRP) 2, but reports from field tests have questioned their sensitivity and reliability. We hypothesize that the variability in the results of PfHRP2-based RDTs is related to the variability in the target antigen. We tested this hypothesis by examining the genetic diversity of PfHRP2, which includes numerous amino acid repeats, in 75 *P. falciparum* lines and isolates originating from 19 countries and testing a subset of parasites by use of 2 PfHRP2-based RDTs. We observed extensive diversity in PfHRP2 sequences, both within and between countries. Logistic regression analysis indicated that 2 types of repeats were predictive of RDT detection sensitivity (87.5% accuracy), with predictions suggesting that only 84% of *P. falciparum* parasites in the Asia-Pacific region are likely to be detected at densities ≤250 parasites/μL. Our data also indicated that PfHRP3 may play a role in the performance of PfHRP2-based RDTs. These findings provide an alternative explanation for the variable sensitivity in field tests of malaria RDTs that is not due to the quality of the RDTs.**

Since the introduction of the first commercially available product 10 years ago [1], rapid diagnostic tests (RDTs) have been widely used for diagnosis of malaria. RDTs have the potential to improve the quality of case management and reduce morbidity and mortality, especially in remote areas. The increasing prevalence of multidrug-resistant malaria and the subsequent change in treatment policies toward more-expensive drug combinations, such as artemisinin combination therapy [2], are increasing the importance of accurate diagnosis before treatment.

Malaria RDTs are lateral-flow immunochromatographic tests that detect specific antigens produced by malaria parasites [3]. At least 25 branded RDT products are now commercially available; some tests detect *Plasmodium falciparum* only, whereas others detect *P. falciparum* plus 1 or more other species of human *Plasmodium*. The major target antigens in RDTs for detection of *P. falciparum* are *P. falciparum* histidine-rich protein (PfHRP) 2, *Plasmodium* lactate dehydrogenase (pLDH), and aldolase.

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**Table 1. Origins of *Plasmodium falciparum* lines and isolates and their detection limits (parasitized erythrocytes per microliter), determined by use of *P. falciparum* histidine-rich protein 2 (PfHRP2)–based rapid diagnostic tests.**

| Region, country/island of origin | Line/isolate | Detection limit | |
|---|---|---|---|
| | | ParaCheck | ICT |
| Africa | | | |
| Burkino Faso | CARDI | … | … |
| Cameroon | CM232 | … | … |
| | CM252 | … | … |
| Gambia | FCR3 | 500 | 250 |
| Ghana | DORCAE | | … |
| | HD1 | … | … |
| Liberia | TRACI | … | … |
| Nigeria | WL (b) | … | … |
| Sudan | 106/1 | … | … |
| | 123/4 | … | … |
| | 105/7 (h) | … | … |
| Uganda | PALO ALTO (d) | … | … |
| Zambia | ZM105 | … | … |
| Pacific | | | |
| Indonesia | ARSO1 (b) | … | … |
| | ARSO2 (b) | … | … |
| Malaysia | CAMP (d) | 250 | 250 |
| Philippines | | | |
| Isabela | PHIL64 | … | … |
| | PHIL66 (g) | … | … |
| | PHIL67 (g) | … | … |
| Mindanao | MIN2 (e) | … | … |
| | MIN3 (e) | … | … |
| | PHIL42 | … | … |
| Morong | PH1 (j) | 250–500 | 250–500 |
| | PH3 (j) | 125 | 125 |
| | PH4 | 1000 | 1000 |
| Palawan | PHIL2 (k) | … | … |
| | PHIL4 (a) | … | … |
| | PHIL6 (k) | … | … |
| | PHIL9 | … | … |
| | PHIL11 (a) | … | … |
| | PHIL12 (a) | … | … |
| | PHIL16 (f) | … | … |
| | PHIL20 | … | … |
| | PHIL21 | … | … |
| | PHIL23 (a) | … | … |
| | PHIL32 (l) | … | … |
| | PHIL35 (l) | … | … |
| Papua New Guinea | | | |
| Bougainvile | AN101 | … | … |
| | AN143 | 500 | 250–1000 |
| | AN185 | … | … |
| Papua New Guinea | FCQ30 | … | … |
| | FCQ33 (c) | 125 | 125 |
| | FCQ41 | 125 | 65 |
| | FCQ49 (c) | 250 | 250 |
| | FCQ64 | 250 | 250 |
| | FCQ79 | … | … |
| | D10 | 1000 | 1000 |
| Solomon Islands | N70 (i) | 250 | 500 |
| | N71 | … | … |
| | N73 (f) | 125 | 125 |
| | SJ15 (h) | 125–500 | 250 |
| | SJ44 (i) | 250 | 500 |
| | S55 | 250 | 250 |
| | S99 (f) | 125 | 125 |
| Southeast Asia | | | |
| Cambodia | CB16 (d) | … | … |
| Vietnam | V2 (m) | … | … |
| | V3 (m) | … | … |
| | V8 | … | … |
| | V9 | … | … |
| | V11 | … | … |
| Thailand | GA3 | 125 | 125 |
| | INDO20 | … | … |
| | INDO21 | 500 | 250 |
| | K1 | … | … |
| | TM92-C734 | … | … |
| | TM91C32B | 125–250 | 125–250 |
| | W2 | 65 | 65 |
| South America | | | |
| Brazil | ALR | … | … |
| | PX403 | … | … |
| | PX413 | … | … |
| | RCS | … | … |
| | S34/89 | … | … |
| Ecuador | ECU1110 | … | … |
| Honduras | HB3 | … | … |
| Peru | PC17 | … | … |

*(continued)*

**Table 1.** ***(Continued.)***

**NOTE.** Letters in parentheses (a–m) indicate isolates with identical *pfhrp2* sequences; the same letter represents the same sequence. Ellipses indicate that the test was not performed.

Several *P. falciparum* RDTs have been tested in the field. Some studies have reported the sensitivity of RDTs to be similar to that commonly achieved by microscopy (~100 parasites/$\mu$L) [1, 4, 5], whereas others have reported sensitivities well below the level required for operational use [6–15]. There is little consistency in the results obtained for individual products or for the same product tested in different locations [1, 6, 8, 11, 13–19]. Although reports of malaria RDTs failing to detect infections with high-level parasitemia exist [8, 10, 14, 15, 20, 23], most of the variation reported has been at relatively low-level parasitemia (100–500 parasites/$\mu$L) [1, 9, 12, 14, 17, 21–25], which usually results in clinical malaria in nonimmune individuals. The causes for such variation have not been elucidated.

Many factors may affect the performance of malaria RDTs. These include parasite factors (species and level of parasitemia,

**Table 2. Code and amino acid repeats observed in *Plasmodium falciparum* histidine-rich protein (PfHRP) 2 and PfHRP3.**

| Code | Repeat sequences | Antigens observed | |
|---|---|---|---|
| | | PfHRP2 | PfHRP3 |
| 1 | AHHAHHVAD | + | + |
| 2 | AHHAHHAAD | + | + |
| 3 | AHHAHHAAY | + | − |
| 4 | AHH | + | + |
| 5 | AHHAHHASD | + | − |
| 6 | AHHATD | + | − |
| 7 | AHHAAD | + | + |
| 8 | AHHAAY | + | − |
| 9 | AAY | + | − |
| 10 | AHHAAAHHATD | + | − |
| 11 | AHN | + | − |
| 12 | AHHAAAHHEAATH | + | − |
| 13 | AHHASD | + | − |
| 14 | AHHAHHATD | + | − |
| 15 | AHHAHHAAN | − | + |
| 16 | AHHAAN | − | + |
| 17 | AHHDG | − | + |
| 18 | AHHDD | − | + |

variability in parasite antigen structure, and persistence of the antigen) and test factors (condition of the RDT, the technique used to perform the test, and the interpretation of the test results). One largely unexplored factor is variability within the parasite antigen being detected by the RDT. This includes presence or absence of the target epitope or variation in the number of epitopes present in a particular parasite isolate. Genetic diversity may be particularly important for PfHRP2-based RDTs, since the antigen consists of a number of alanine- and histidine-rich amino acid repeats [26] and varies in size between parasite strains [27]. Comparison of the PfHRP2 sequences from several parasite strains has shown differences in the number of tri- and hexapeptide repeat units and rare amino acid variants [26, 28, 29]. An additional report showed that the amino acid sequence of PfHRP2 in a Chinese isolate was different from that in South American (7G8) and Gambian (FCR3) isolates [30]. To date, however, no systematic examination of the diversity in PfHRP2 across geographically diverse regions has been performed, nor has any relationship between antigenic diversity and RDT detection sensitivity been examined. In this article, we report the genetic diversity of the *pfhrp2* gene and translated protein in a collection of *P. falciparum* lines and isolates originating from 19 countries and assess the relationship between the *pfhrp2* diversities and the sensitivities of 2 PfHRP2-based RDTs.

## MATERIAL AND METHODS

***Parasite lines and isolates.*** A total of 75 *P. falciparum* lines and isolates from 19 countries were analyzed (table 1). Fifty-seven of these were laboratory lines and isolates either stored in our laboratories [31–34] or provided by N. Anh and K. Andrews. The remaining 18 parasite isolates were collected from the Philippines during RDT field trials. These trials were approved by the Ethics Review Board, Research Implementation and Development Office, College of Medicine, University of the Philippines, Manila. Blood samples were collected by finger prick and stored as dried filter spots.

***DNA isolation.*** Genomic DNA was isolated from cryopreserved laboratory lines or cultures by use of a method described elsewhere [35] and from dried filter spots by use of a QIAblood kit (QIAGEN), in accordance with the manufacturer's instructions.

***Polymerase chain reaction (PCR) amplification of* pfhrp2 *and* pfhrp3.** The primers Pfhrp2-F1 (5′-CAAAAGGACTTAATTTAAATAAGAG-3′) and Pfhrp2-R1 (5′-AATAAATTTAATGGCGTAGGCA-3′) were designed to anneal to the 5′ and 3′ ends of exon 2 of *pfhrp2*. For amplification of *pfhrp2* from culture-adapted material, a single round of 40 amplification cycles was performed by use of 2 mmol/L $Mg_2Cl$, 0.2 mmol/L each deoxynucleoside triphosphate (Promega), 75 ng of each primer, 1.25 U of AmpliTaq Gold (PE Applied Biosystems), and 1 $\mu$L of DNA, under the following cycling conditions: 94°C for 10 min, followed by 94°C for 50 s, 55°C for 50 s, and 70°C for 1 min. For samples extracted from filter papers, a seminested amplification was performed by use of the primers Pfhrp2-F2 (5′-ATTATTACACGAAACTCAAGCAC-3′) and Pfhrp2-R1, under cycling conditions identical to those used in the first round. The same procedures and conditions were used to amplify the *pfhrp3* gene by use of the primers Pfhrp3-F1 (5′-AATGCAAAAGGACTTAATTC-3′), Pfhrp3-R1 (5′-TGGTGTAAGTGATGCGTAGT-3′), and Pfhrp3-F2 (5′-AAATAAGAGATTATTACACGAAAG-3′).

***DNA sequencing and translation of DNA sequences.*** PCR products were purified by use of spin columns (QIAGEN) and were used in a standard dye-terminator DNA sequencing reaction (ABI). Nucleotide sequences were translated to corresponding amino acids. Each type of amino acid repeat was identified and given a numeric code (1–18). A bar code specific to each isolate was developed by listing the numeric code in the order of the repeats. The sequences reported in this article have been deposited in the GenBank database (accession numbers for *pfhrp2,* AY816237–AY816310; accession numbers for *pfhrp3,* AY821805–AY821825).

***In vitro cultivation and dilution of parasites.*** Twenty-one

**Table 3. Sequence of *Plasmodium falciparum* histidine-rich protein 2 (PfHRP2) represented by bar codes.**

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

**Table 4. Comparison of the range of total no. of repeats and each individual repeat in *Plasmodium falciparum* histidine-rich protein 2, in parasites from different geographical areas.**

| Region, country/island of origin | No. | Total | No. of total and individual repeats | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 |
| Africa | | | | | | | | | | | | | | | | |
| Burkino Faso | 1 | 30 | 1 | 12 | 0 | 1 | 0 | 3 | 8 | 2 | 0 | 2 | 0 | 1 | 0 | 0 |
| Cameroon | 2 | 32–35 | 5 | 11–12 | 1–2 | 0 | 1 | 4–5 | 4–8 | 1 | 0 | 1–2 | 1–2 | 1 | 0–1 | 0–1 |
| Gambia | 1 | 34 | 2 | 9 | 1 | 0 | 2 | 7 | 9 | 2 | 0 | 1 | 0 | 1 | 0 | 0 |
| Ghana | 2 | 31–33 | 1–3 | 12–14 | 1–3 | 0–1 | 0–1 | 3–4 | 6–8 | 1 | 0 | 1–2 | 0 | 1 | 0 | 0 |
| Liberia | 1 | 33 | 3 | 12 | 1 | 2 | 1 | 3 | 8 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Nigeria | 1 | 33 | 1 | 9 | 1 | 0 | 2 | 7 | 9 | 2 | 0 | 1 | 0 | 1 | 0 | 0 |
| Sudan | 3 | 28–31 | 1–5 | 7–11 | 1 | 1 | 1–2 | 2–6 | 5–9 | 1–2 | 0 | 0–2 | 0 | 1 | 0 | 0 |
| Uganda | 1 | 29 | 3 | 13 | 2 | 0 | 0 | 2 | 7 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| Zambia | 1 | 31 | 3 | 13 | 2 | 0 | 1 | 3 | 6 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Pacific | | | | | | | | | | | | | | | | |
| Indonesia | 2 | 33 | 1 | 9 | 1 | 0 | 2 | 7 | 9 | 2 | 0 | 1 | 0 | 1 | 0 | 0 |
| Malaysia | 1 | 29 | 3 | 13 | 2 | 0 | 0 | 2 | 7 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| Philippines | 21 | 25–35 | 1–4 | 6–15 | 0–3 | 0–3 | 0–2 | 2–4 | 1–10 | 1–2 | 0–1 | 0–2 | 0–1 | 1 | 0 | 0 |
| Papua New Guinea | 9 | 24–38 | 1–3 | 7–15 | 1–2 | 0–2 | 1–3 | 2–7 | 2–9 | 1–2 | 0 | 1–2 | 0 | 1 | 0 | 0–1 |
| South America | | | | | | | | | | | | | | | | |
| Brazil | 5 | 31–33 | 2–4 | 9–12 | 2 | 0 | 0–2 | 2–5 | 8–10 | 0–1 | 0 | 0–3 | 0 | 1 | 0 | 0 |
| Ecuador | 1 | 33 | 5 | 12 | 1 | 0 | 1 | 4 | 7 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Honduras | 1 | 32 | 5 | 12 | 1 | 0 | 1 | 2 | 8 | 1 | 0 | 1 | 0 | 1 | 0 | 0 |
| Peru | 1 | 32 | 4 | 13 | 1 | 1 | 0 | 2 | 6 | 1 | 0 | 2 | 0 | 1 | 1 | 0 |
| Southeast Asia | | | | | | | | | | | | | | | | |
| Cambodia | 1 | 29 | 3 | 13 | 2 | 0 | 0 | 2 | 7 | 1 | 0 | 0 | 0 | 1 | 0 | 0 |
| Vietnam | 5 | 30–36 | 2–6 | 13–15 | 1–2 | 1–2 | 1 | 3–4 | 2–7 | 1 | 0 | 1–2 | 0 | 1 | 0 | 0 |
| Solomon Islands | 7 | 30–36 | 2–4 | 11–16 | 1–2 | 0–1 | 1–2 | 3–7 | 4–9 | 1 | 0 | 1–2 | 0–1 | 1 | 0 | 0–1 |
| Thailand | 7 | 27–36 | 1–5 | 10–19 | 1–3 | 0–3 | 0–1 | 2–4 | 1–6 | 0–2 | 0 | 0–2 | 0 | 1 | 0–1 | 0–1 |
| Overall | | 25–38 | 1–6 | 6–19 | 0–3 | 0–3 | 0–3 | 2–7 | 1–10 | 0–2 | 0–1 | 0–3 | 0–2 | 1 | 0–1 | 0–1 |

parasite lines were cultured in vitro by use of conditions modified from those reported elsewhere [36]. Parasite cultures were synchronized by repeatedly removing mature stages by use of 5% sorbitol [37]. The density of ring-infected erythrocytes was determined by multiplying the proportion of ring-infected erythrocytes by the number of erythrocytes per microliter. Parasite-blood suspensions representing 100,000, 20,000, 4000, 2000, 1000, 500, 250, 125, and 65 ring-infected cells/$\mu$L were prepared by serial dilution of the culture stock in uninfected human erythrocytes at a hematocrit of 40%.

***RDT.*** A total of 5 $\mu$L of each parasite-blood suspension was spotted onto the ParaCheck Pf (Orchid Biomedical Systems) and ICT Malaria Pf (R&R Marketing) test kits, followed by addition of the buffer, as described in the product instructions. Results were recorded at the time recommended by the manufacturer. The detection limit/sensitivity for each parasite line was recorded as the minimum level of parasite density that gave a positive result. When multiple culture and tests of the same isolate were conducted, the mean of detection limits was used in the analysis.

***Statistical analyses.*** Differences in the presence or absence of identified repeats in isolates from different countries/regions were tested by use of $\chi^2$ analysis. Differences in the mean number of repeats of each type and the total number of repeats between countries/regions were tested by use of multivariate analysis; Tukey's test was used for post-hoc multiple comparisons. Binary logistic regression was used to examine the ability of the repeat sequences to predict whether a test result was sensitive (detection limit, $\leq$250 parasites/$\mu$L) or not sensitive (detection limit, >250/$\mu$L).

## RESULTS

***Variation in PfHRP2.*** PCR-amplified *pfhrp2* fragments from the isolates varied in size from 600 to 960 bp (data not shown). Much of this variation was caused by differing numbers of 27-, 18-, and 9-bp repeats. When translated into amino acid sequences, PfHRP2 consisted of varying numbers of 9-, 6-, and 3-aa repeats. A total of 14 different amino acid repeats were identified from the PfHRP2 sequences of 74 isolates (table 2). One parasite line (D10) did not express PfHRP2, because of a gene deletion [38]. Fifty-six unique PfHRP2 sequences were identified; 43 were seen only once, whereas the remaining 13 sequences were seen in >1 parasite isolate. Nine of these 13 shared sequences were shared by isolates from the same country, whereas 4 were shared by isolates from different countries (table 1).

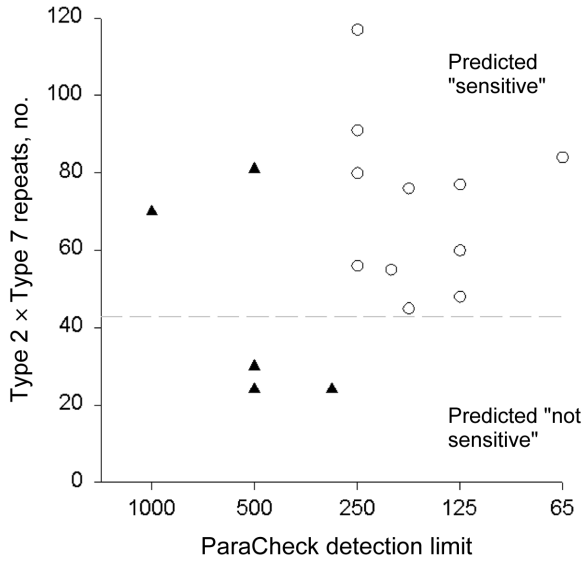


**Figure 1.** Isolates tested and predicted to be sensitive (detectable at densities ≤250 parasites/μL) and not sensitive (detectable at densities >250 parasites/μL) to rapid diagnostic tests, by use of the binary logistic regression model $ln[P/(1-P)] = 2.5035 + 0.0583 \times (\text{type } 2 \times \text{type } 7)$. Circles represent "sensitive" isolates, whereas triangles represent "nonsensitive" isolates. The horizontal dotted line represents the predicted cutoff for sensitivity.

***Overall variation.*** All distinct PfHRP2 sequences started with the type 1 repeat (AHHAHHVAD) and ended with the type 12 repeat (AHHAAAHHEAATH). The composition, number, and order of the repeat types in PfHRP2 differed between individual parasite isolates (table 1 and supplemental data in table 3). A relatively conserved motif of types 7, 8, 2, and 7 repeat was present in the central region of the sequence in 87.8% (65/74) of the isolates sequenced. Of the 9 isolates that did not have this motif, 7 (9.5%) were lacking only the first or last type 7 repeat.

To analyze whether significant differences in PfHRP2 structure existed between parasites collected from different areas, countries with small sample numbers (≤5) were grouped into geographic regions (table 1). Countries with sample numbers >5 were included directly in the analysis.

***Variation in frequency of repeats.*** Type 1 (AHHAHHVAD), type 2 (AHHAHHAAD), type 6 (AHHATD), type 7 (AHHAAD), and type 12 (AHHAAAHHEAATH) repeats were observed in 100% of the isolates sequenced. Several of the repeats occurred in only a few isolates: type 9 (AAY) in 1 isolate from the Philippines, types 11 and 13 (AHN and AHHASD) in 3 samples each, and type 14 (AHHAHHATD) in 5 isolates (table 4). No differences in the presence/absence of each repeat were detected between geographic areas ($P > .05$).

***Variation in the number of repeats.*** The total number of repeats and the number of each repeat within PfHRP2 varied between isolates, both within and between countries (table 4). When the number of repeats was compared between geographic areas, differences were seen for 3 types of repeats ($P < .01$). Isolates from Papua New Guinea (PNG) had significantly fewer type 1 repeats (mean, 1.89) than did isolates from South America (mean, 3.75) and Southeast Asia (mean, 3.83) ($P = .002$). For type 6 repeats, isolates from Thailand (mean, 2.86) and the Philippines (mean, 2.90) had significantly fewer repeats than did isolates from PNG (mean, 4.78) and the Solomon Islands (mean, 4.86) ($P = .002$). Finally, for type 7 repeats, isolates from Thailand (mean, 4.00) had significantly fewer repeats than did isolates from South America (mean, 8.00) ($P = .007$).

***RDT detection sensitivity.*** All 21 isolates that had been cultured in vitro were detected by both commercially available RDTs at densities ≥1000 parasitized erythrocytes/μL (table 1). Although the D10 clone was tested and detected by both RDTs, it has been excluded from the analysis described hereafter because of the deletion of the *pfhrp2* gene. The average detection limit was used for isolates with identical PFHRP2 sequences, resulting in 16 unique RDT results. There was good concordance between the 2 RDTs, with 69% (11/16) of the isolates tested having the same detection limit. The 6 isolates that had different readings differed by no more than 1 dilution.

The ParaCheck RDT detection sensitivity was classified as sensitive (detection level, ≤250 parasitized erythrocytes/μL) or not sensitive (>250 parasitized erythrocytes/μL). By use of binary logistic regression, a significant model ($P = .025$) predicting detection sensitivity that was based on the number of type 2 × type 7 repeats was developed: $ln[P/(1-P)] = 2.5035 + 0.0583(\text{type } 2 \times \text{type } 7)$, where $P$ is the probability of an isolate giving a sensitive test result; $P > .5$ was the cutoff for a sensitive test result (figure 1). This model had an accuracy of 87.5% (Nagelkerke $R^2 = 0.353$) and predicted an isolate to be sensitive if the number of type 2 × type 7 repeats was >43. No association between detection sensitivity and the position of types within a sequence, the length of runs of type 2 and 7 repeats, or the pattern of the type 2 repeat relative to that of the type 7 repeat could be found.

The regression model developed from the data on 21 isolates was used to predict the detection sensitivity of all 74 isolates on the basis of their PfHRP2 sequences. Overall, 16% (12/74) of isolates were predicted to not be detected by the RDTs at a parasitemia level ≤250 parasites/μL (table 5). Closer examination revealed that all the isolates predicted to be nonsensitive (including the ones actually tested) originated from the Asia-Pacific region, specifically the Philippines, PNG, Vietnam, and Thailand. The proportion of isolates from these regions that were predicted to not be detected by the RDTs at densities ≤250 parasitized erythrocytes/μL ranged from 17% to 33% (table 5), with an overall nonsensitive rate for the Asia-Pacific region of 23% (12/51).

***Possible role of PfHRP3 in RDT detection sensitivity.*** We

**Table 5. Prediction of rapid diagnostic test detection sensitivity by use of the regression model.**

| Region | Nonsensitive, no. (%) | Sensitive, no. | Total, no. |
|---|---|---|---|
| Africa | 0 | 13 (100) | 13 |
| Pacific | 0 | 3 (100) | 3 |
| Philippines | 6 (29) | 15 (71) | 21 |
| Papua New Guinea | 3 (33) | 6 (67) | 9 |
| South America | 0 | 8 (100) | 8 |
| Southeast Asia | 1 (17) | 5 (83) | 6 |
| Solomon Islands | 0 | 7 (100) | 7 |
| Thailand | 2 (29) | 5 (71) | 7 |
| Total | 12 (16) | 62 (84) | 74 |

**NOTE.** The detection limit for sensitive isolates was ⩽250 parasitized erythrocytes/$\mu$L, and that for nonsensitive isolates was >250 parasitized erythrocytes/$\mu$L.

tested D10 using 2 PfHRP2-based RDTs and found that this clone gave positive test results at parasitemias ⩾1000 parasites/$\mu$L. PCR using *pfhrp2*-specific primers failed to amplify a product, whereas amplification using *pfmsp1*-, *pfmsp2*-, and *pfhrp3*-specific primers amplified the corresponding products (data not shown). This is consistent with a gene deletion [38] and suggests that other antigens may be detected by these RDTs.

One candidate antigen is PfHRP3, since significant homology exists between PfHRP2 and PfHRP3 and since monoclonal antibodies (MAbs) against PfHRP2 cross-react with PfHRP3 [27]. *pfhrp3* from the isolates that had been cultured and tested for performance of RDTs was amplified and sequenced. Like PfHRP2, PfHRP3 consists of alanine- and histidine-rich repeats. Eight types of repeats were observed in PfHRP3, of which types 1, 2, 4, and 7 were identical to those in PfHRP2 (table 1). The majority of the sequences comprised 2 sections of repeats. The first started with a type 1 repeat and ended with a type 7 repeat. This was followed by a stretch of a nonrepetitive amino acid sequence and then the second section of repetitive sequence, which predominantly consisted of type 17 and type 18 repeats and ended with a type 4 repeat (table 2 and supplemental data in table 6). Although there was also variation in the composition and order of the repeats, the number of type 7 and type 2 repeats was much less variable than that observed in PfHRP2 (table 2 and supplemental data in table 6).

## DISCUSSION

Malaria RDTs offer great potential for rapid and accurate diagnosis of malaria infections, which could lead to prompt and appropriate treatment of the disease. Achieving acceptable sensitivity and specificity in malaria RDTs is imperative if these devices are to be widely used. Unfortunately, the genetic diversity within the antigens targeted by the antibodies used in the RDTs has the potential to affect their sensitivity.

In this article, we have reported extensive diversity in PfHRP2 found in parasite isolates from around the world. Of the 14 different amino acid repeats that we identified in PfHRP2, only 5 were found to be present in all isolates. The remaining repeats tended to be present in either most or very few of the isolates. If MAbs used in any PfHRP2-based RDT recognize epitopes residing in repeats that are not universally present, the test could miss a significant proportion of parasites, giving false-negative results. For the 2 RDTs that we tested, all isolates gave a positive result at parasite densities ⩾1000 parasites/$\mu$L. A significant relationship was observed between the number of type 2 and type 7 repeats and the ability to detect low parasite densities (⩽250 parasitized erythrocytes/$\mu$L), with higher numbers of type 2 and type 7 repeats correlating with better sensitivity. This result suggests that it is likely that the epitopes recognized by these RDTs reside within or between type 2 and type 7 repeats. Although type 2 and type 7 repeats were found in all isolates tested, our results indicate the numbers of these repeats varied the most: 6 to 19 and 1 to 10 for types 2 and 7, respectively. These data suggest that RDTs would fail to detect a substantial number of isolates with fewer type 2 and type 7 repeats at parasite densities ⩽250 parasitized erythrocytes/$\mu$L. Although this level of parasitemia is generally not life threatening, it would produce clinical symptoms in a high proportion of nonimmune individuals. If parasitemia is undetected and the patient is left untreated, he or she may develop severe complications.

There have been reports of *pfhrp2* gene deletions in some laboratory clones (D10 and W2Mef) [38–40]. We observed only *pfhrp2* gene deletions in these 2 laboratory clones, indicating that deletion events probably occur at low frequencies. An alternative explanation for the lack of isolates with a *pfhrp2* deletion could be that such a gene deletion results in a loss of fitness of the parasite. More-extensive field surveys are needed to establish the frequency and extent of *pfhrp2* deletion.

Detection of the D10 clone by the 2 RDTs we tested suggests that the antibodies present in these PfHRP2-based RDTs cross-react with other parasite antigens. The most likely candidate antigen for this cross-reactivity is PfHRP3. Wellems et al. reported a 85%–90% homology in nucleotide sequence–flanking repeats between *pfhrp2* and *pfhrp3* and an amino acid substitution of D/N in the major repeat of AHHAAD/N in 1 parasite line [26]. Our results further demonstrate that *pfhrp3* encodes 4 types of alanine- and histidine-rich amino acid repeats that are identical to those present in PfHRP2 (types 1, 2, 4, and 7) and 4 that are different. It is likely that either some or all of

**Table 6. Sequences of *Plasmodium falciparum* histidine-rich protein 3 (PfHRP3) represented by bar codes.**

The table is available in its entirety in the online edition of the *Journal of Infectious Diseases.*

the epitopes detected by antibodies used in the RDTs also exist in PfHRP3. Indeed, the results of our regression analysis suggest that type 2 and type 7 repeats may be involved in the observed cross-reactivity. Varying degrees of cross-reactivity between PfHRP2 and PfHRP3 have also been reported for several PfHRP2 MAbs: 2G12, MAb87, and 1D6 [27]. Antisera generated against peptides of PfHRP2 and PfHRP3 predominantly recognize the corresponding protein but also weakly recognize the others [41]. It is also possible that other parasite histidine-rich proteins may be recognized by PfHRP2 MAbs. Overall, this cross-reactivity was strong enough to enable the detection of D10 parasites at a parasitemia $\geqslant$1000 parasites/$\mu$L, providing a "safety net" in the use of RDTs when parasites have a *pfhrp2* deletion. The cross-reactivity is also likely to modulate the effect of the genetic diversity exhibited in PfHRP2 on the detection sensitivity of RDTs, since PfHRP3 is less variable than PfHRP2. A second factor that may modulate the effect of PfHRP2 genetic diversity is that the RDTs use 2 antibodies: 1 acting as a signal antibody and 1 acting as a capture antibody. Both antibodies recognize epitopes on PfHRP2. If 2 antibodies recognize different epitopes, then a reduced number of one epitope may be compensated by a larger number of the second epitope. The combined effect of cross-reactivity between PfHRP2 and PfHRP3 and the inclusion of 2 different PfHRP2 antibodies in RDTs may assist with the reliable detection of parasites at densities >1000 parasites/$\mu$L. Genetic diversity in PfHRP2 appears to mainly affect detection at parasitemias <1000 parasites/$\mu$L.

The majority of the commercially available RDTs are based on detection of PfHRP2 and identify only *P. falciparum*. There is no published information available on the specific target epitopes of the antibodies used in any of these RDTs or on the 6 published MAbs that are directed against PfHRP2 [38, 41–43]. Because of the limited supply of MAbs, it is very likely that most of the PfHRP2-based RDTs use similar or identical antibodies. Therefore, we expect that the effect of genetic diversity on the performance of the 2 PfHRP2-based RDTs reported here applies to the majority of other commercially available PfHRP2-based RDTs. Our study highlights the urgency in defining the epitopes recognized by these MAbs. Such data will allow us to use the PfHRP2 sequence information to better predict the potential effect of genetic diversity on the performance of these RDTs and to design more-sensitive RDTs that use more-sensitive or multiple MAbs.

On the basis of the PfHRP2 sequences and our laboratory test results, we predict that ~23% of parasites in the Asia-Pacific region are not detectable at densities $\leqslant$250 parasites/$\mu$L. Hence, we would expect more false-negative results or RDT failures in this region than in other regions. However, more-comprehensive sequence analysis with larger sample numbers for each country is needed to provide a better estimation of the distribution of these "nonsensitive" parasites. An assessment of variation in the other target antigens of malaria RDTs, such as pLDH and aldolase, also is warranted.

Our findings provide a possible explanation for some of the variable sensitivity reported in field tests of malaria RDTs that is not due to the quality of the RDTs. This does not undermine the importance of quality control in the manufacturing process or in the transport and storage of the devices, since poor quality control and deterioration during storage could lower sensitivity further. Furthermore, our findings on the relationship between PfHRP2 genetic diversity and RDT sensitivity present a serious challenge to future testing and quality control of these devices. It may be appropriate to use a panel of parasite isolates with varying PfHRP2 sequences for initial product testing and evaluation. The genetic background of the parasites should be taken into account when these devices are tested in field settings or when sensitivities are compared between different settings or assessed against locally endemic parasites.

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